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(54) Glycolytic promoters for regulated protein expression: protease inhibitor.

(57) Promoters associated with expression of specific enzymes in the glycolytic pathway are used for expression of alien DNA, particularly yeast promoters known to provide high enzyme levels of enzymes in the glycolytic pathway are employed for expressing a mammalian protein, such as alpha-1-antitrypsin. The promoters include promoters involved in expression of pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructose kinase, and aldolase, as well as the glycolytic regulation gene. Particularly, the glycolytic regulation gene can be used in conjunction with promoters in the glycolytic pathway for regulated production of desired proteins.

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GLYCOLYTIC PROMOTERS FOR REGULATED PROTEIN EXPRESSION: PROTEASE INHIBITOR

The ability to obtain expression of foreign, i.e., exogenous, DNA in unicellular microorganisms provided the opportunity to conveniently prepare long polypeptide chains of interest. Almost immediately, varied polypeptides, such as the small hormone somatostatin and more sophisticated polypeptides, such as insulin, interferons, thymosin and a variety of vaccines having capsid proteins, were prepared and reported in the literature. For the most part, the initial work was performed in the bacterium E. coli which had been the subject of intensive study because scientists were familiar with many aspects of its genetic structure and properties. Initial attention was therefore directed to producing foreign proteins in E. coli. Once the ability to employ E. coli as a host was established, the limitations and disadvantages of employing E. coli encouraged the use of other hosts.

One host which appeared to be particularly attractive because it lacked many of the shortcomings of E. coli was yeast. However, yeast is a eukaryote and, therefore, has a more sophisticated genetic system. Furthermore, less is known about the yeast genome than is known about E. coli. In order to use yeast as a host for the production of proteins foreign to yeast, a number of discoveries are required, and new materials must be made available.

Initially, a replication system was required which provided stability in yeast, either as an extrachromosomal element or by integration into the yeast chromosome. In addition, the regulatory functions concerned with transcription and expression had to be developed in order to allow for expression of the desired protein. There was also the uncertainty whether foreign DNA sequences would be transcribed and translated and, if expressed, whether the resulting polypeptides would survive in the yeast cell. Also remaining to be determined was the effect of the foreign proteins on the viability of the yeast cell, such as the effect of recombinant DNA (RDNA) on mitosis, sporulation and vegetative growth.

There have, therefore, been substantial efforts to develop novel RDNA systems in yeast, which will allow for regulated expression of a protein of interest, as well as highly efficient production of such proteins.

Hitzeman et al., J. Biol. Chem., 255:12073-12080 (1980) describe a plasmid having a yeast 3-phosphoglycerate kinase (PGK) gene and accompanying regulatory signals capable of expression in yeast. Other references of interest include Clifton, et al., Genetics, 88:1-11 (1978); Clark and Carbon, Cell, 9:91-99 (1976); Thomson, Gene, 1:347-356 (1977); Holland and Holland, J.Biol. Chem., 254:5466-5474 (1979); Holland and Holland, ibid. 254:9830-9845 (1979); Nasmyth and Reed, Proc. Nat. Acad. Sci., 77:2119-2123 (1980); Broach, et al., Gene, 8:121-133 (1979); and Williamson, et al., Nature, 283:-214-216 (1980).

In the accompanying FIGURES:

FIGS. 1A and 1B are cDNA sequences of two forms of genes coding human alpha-1-antitrypsin.

FIG. 2 illustrates the restriction maps of plasmids CTEA32 and CAT1.

FIG. 3 is a diagram of the electrophoresis chromatogram showing purified alpha-1-antitrypsin produced according to the present invention.

FIG. 4 illustrates the restriction map of plasmid C1/1.

FIG. 5 illustrates the DNA sequence of the multiple restriction site of pUC13.

FIG. 6 illustrates the restriction map of plasmid pUCα1 containing the DNA sequence from FIG. 1.

FIG. 7 is the restriction map of plasmid HAT4.

Novel yeast promoters are provided which control the transcription of genes in the glycolytic pathway and which find use in the regulated production of proteins foreign to the yeast. Promoters of particular interest include the promoters for triose phosphate isomerase, pyruvate kinase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructo kinase, and aldolase, as well as the glycolytic regulatory gene. The protease inhibitor, mammalian alpha-1-antitrypsin, is expressed using the promoter for triose phosphate isomerase.

Methods and compositions are provided for regulated efficient expression of alien or foreign DNA in a yeast host. (Alien or foreign DNA is DNA not naturally occurring in the wild type particularly from a different species and which does not normally exchange genetic information with the host.) Novel promoters are employed which are involv d in th glycolytic pathway and provide for high levels of protein production, so that a

substantial proportion of the total protein produced by the yeast cells can be dedicated to the protein of interest. In addition, regulatory mechanisms associated with regulation of production of the glycolytic enzymes are achieved, so that production of the desired products may be modulated. Furthermore, viable cells can be maintained to enhance the efficiency and amount of expression.

The promoters of interest are particularly those promoters involved with expression of triose phosphate isomerase, pyruvate kinase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructo kinase, and aldolase, which are controlled by the glycolytic regulation gene GCR1. The genes of the glycolytic pathway include hexokinase 1 and 2 (HXK1,2); phosphoglucose isomerase (PGI), triose phosphate isomerase (TPI); phosphoglycerate kinase (PGK), phosphoglycerate mutase (GPM), pyruvate kinase (PYK) phsphofructo kinase (PFK), enolase (ENO); fructose 1,6-diphosphate aldolase (FDA); glyceraldehyde 3-phosphate dehydrogenase (PGK); and glycolysis regulation protein (GCR).

The promoters may be obtained by employing a gene bank having large fragments of yeast DNA. By introducing the fragments into appropriate vectors, particularly shuttle vectors having replicons for prokaryotes and yeast, one can readily amplify and clone the yeast DNA in a bacterium and then introduce the yeast DNA into mutant yeast cells for complementation. In this manner, yeast fragments can be identified which complement auxotrophic lesions or mutations in a yeast host.

Of particular interest, is where the host is auxotrophic in both the glycolytic pathway step of interest and a separate biochemical pathway, which is complemented by a

marker in the vector. Once having established a DNA segment having the desired gene, one may reclone by various techniques to shorten the DNA segment and provide for a segment which is primarily the gene of interest in conjunction with its regulatory signals for transcription and expression.

In order to retain the promoter, it is essential that the initiator methionine be determined and this codon be used for developing the strategy for introducing the alien DNA downstream from the promoter. Various techniques can be employed for providing a site for introduction of the alien DNA so as to be under the regulatory control of the promoter in the glycolytic pathway.

Where a restriction site is conveniently adjacent to the initiator methionine codon, the glycolytic gene may be cleaved at that site and the DNA chewed back with Bal31 for varying periods of time, so as to chew into or past the initiator methionine codon or retain the initiator methionine codon.

Where there is no convenient restriction site, other splicing techniques such as primer repair may be employed. Also, by employing <u>in vitro</u> mutagenesis, one can introduce a restriction site adjacent the initiator methionine, which encodes for the initial amino acids of the desired protein. In each instance, a linearized DNA segment is obtained having the intact promoter for the glycolytic product and normally includes other DNA sequences, such as an intact replicon, one or more markers, and the like.

Exemplary of the above procedure is the development of a vector having the promoter for the <u>TPI</u>1 gene. An exemplary vector CV13 having the replicons or replica-

tion systems from pBR322 and 2μ-plasmid of yeast, as well as the LEU2 gene was employed for insertion of a yeast fragment which was shown to have the TPI1 gene. This was achieved by employing double selection with a mutant yeast which was leu⁻, tpi⁻. The TPI1 gene was found to have a unique KpnI site. The vector was cleaved at the KpnI site and then treated with the double stranded exonuclease Bal31 for varying times to chew back the DNA to about the f-met codon. Linkers were then inserted providing desired restriction sites. Alien DNA could then be inserted providing a sequenc having a f-met codon in the appropriate position for initiation. Alternatively, the foreign DNA can be expressed using the f-met codon of the TPI1 gene.

Similar procedures can be performed with the other subject glycolytic genes in order to provide the promoters associated with those genes. The PYK sequence has a convenient XbaI site for restriction, where the few additional bases may be removed, if required, using Bal31 for a short period of time to chew to or through the methionine codon. Of particular interest is the use of the GCR promoter to control the expression of the other genes involved in the glycolytic pathway. By employing the GCR gene, in conjunction with other glycolytic promoters regulating expression of alien DNA, one can turn on and off the other promoters, so as to regulate the expression of the alien DNA. Thus, one can allow vegetative growth to proceed until a desired cell density is achieved, before permitting production of the desired polypeptide.

By employing appropriate auxotrophs, one can further regulat the expression of the polypeptides of interest in choosing the appropriate nutrient medium. Where the chosen promoter is repressed by the particular nutrient because of a metabolic block, a change in the nature of

the nutrient can induce expression. Furthermore, the activity of a number of promoters in the glycolytic pathway can be affected by the repression or activation of expression by the GCR gene or other regulatory controls. Also, the GCR regulatory signals can be used to titrate the polypeptide functioning as the regulator for expression of GCR. By having vectors whose copy number can be controlled, one can vary the activity of the wild type GCR gene.

In order to obtain expression, an extrachromosomal element construct will be prepared having a number of sequences defining different functions. One function is the replication system, which forms part of a vector. Another function is a promoter by itself or in conjunction with the alien DNA. Other functions include initiators and terminators of expression. Also, there will be selectable markers.

In developing an appropriate vector, while not necessary, it will be common to have both a replication system for yeast and a replication system for a prokaryote (a shuttle vector). The replication system for yeast may be one which provides for stable maintenance of an extrachromosomal element or one which provides a sufficient lifetime for the DNA in the host, that there is an acceptable probability of integration of the DNA into the host. Integration can be greatly aided by providing for a sequence homologous to the host DNA, so as to provide for recombination. Generally, the homologous sequence will be at least about 800bp usually not more than about 2000bp. Therefore, either integration or an autonomous replication system, such as the use of the ARS1 gene, may be employed to provide for the maintenance of the alien DNA in the yeast host. The replication system which is chosen should provide for a reasonable copy number usually greater than 1, pref-

erably greater than 5. A wide variety of replication systems are available on a wide variety of prokaryotic vectors, such as pBR322, pACYC184, pSC101, pMB9, etc. Alternatively, one or more copies of the DNA construct can be integrated into the host chromosome. The replication systems may also be conditionally regulated, usually being temperature sensitive so that replication can be turned on and off by varying the temperature.

In addition to the replication system, there will also be one or more selectable markers, there usually being at least one marker in addition to the alien DNA, which may serve as a marker. Conventional markers include biocidal markers providing antibiotic resistance and those providing resistance to toxins and heavy metal. Also useful is employing an auxotrophic host and providing prototrophy by complementation. In addition to the conventional selection systems just described, the glycolytic genes of the present invention are particularly desirable markers since they can provide for selection, using sugars as selective substrates, in appropriate mutant host strains.

Other genes may also be inserted into the extrachromosomal element for a variety of purposes. Where integration is desirable in the genome of the host, a homologous sequence for a particular region of the host genome may be included in the extrachromosomal element. Where amplification of one or more sequences is desired, genes known to provide such amplification, such as dihydrofolate reductase genes, which respond to methotrexate stress or metallothionein genes, which respond to heavy metal stress, may be included in the extrachromosomal element, flanked by the DNA regions to be reiterated. Oth r regulatory signals may also be

included, such as centromeres, autonomously replicating segments, etc.

In order to isolate the promoters of interest, clones can be made of yeast chromosomal DNA by random digestion or mechanical shearing of the yeast genome. The presence of the desired gene is then determined by introducing a homogeneous clone of a yeast fragment into an auxotrophic host for complementation. Desirably, the cloning vehicle may have another gene which allows for an additional basis for selection, so that double selection techniques can be used. The mutants are substantially incapable of growing on limited nutrient medium, so that one can select for the presence of the desired glycolytic gene by the choice of medium. After isolating the yeast fragment having the desired gene, the fragment may be subcloned so as to remove superfluous DNA flanking regions and provide for a fragment which is more easily manipulated. The smaller fragment containing the desired gene, of a size less than about 500 base pairs may then be further cloned, restriction mapped and sequenced, so as to provide a useful source for the desired promoters and insertion of the alien DNA. Also, as indicated, the promoters in themselves may be useful, in acting as a titrater for repressor or activator, where it is desirable to modulate the production of a particular enzyme in the yeast host. The alien DNA may be from any source, either naturally occurring or synthetic, either prokaryotic or eukaryotic. Of particular interest are mammalian genes which express a poly(amino acid), that is, polypeptide or protein which has physiological activity. To varying degrees, poly(amino acids) prepared in yeast may be modified by glycosylation, where the glycosylation may not occur or may occur at different sites from the naturally occurring mammalian polypeptide and/or in different degrees with different saccharides. It is

therefore of great interest to be able to prepare polypeptides which are different from the naturally occurring polypeptide by the degree and manner of glycosylation and in many instances may differ in one or more ways as to the amino acid sequences, where there may be deletions of one or more amino acids or substitutions of one or more amino acids. Mammalian genes may come from a wide variety of mammalian sources, such as domestic animals (e.g. bovine, porcine, ovine and equine) and primates e.g. humans and monkeys.

As exemplary of the use of the subject promoters in preparing an active polypeptide composition, as well as being of particular interest for a variety of purposes, a protease inhibitor is described and made. The protease inhibitor has the same or substantially th same amino acid sequence of human alpha-1-antitrypsin and is capable of inhibiting a number of proteolytic enzymes. The human alpha-1-antitrypsin gene appears to reside within a 9.6 kb EcoRI DNA fragment in the human genome. The mature mRNA appears to have about 1400 nucleotides. One human alpha-1-antitrypsin cDNA has the sequence shown in FIG. 1B. The predominant form of human alpha-1-antitrypsin is shown in FIG 1A. Other naturally-occurring forms (polymorphisms) are known.

The sequencing of chromosomal DNA coding for alpha antitrypsin has been described by Kurachi et al., Proc. Natl. Acad. Sci. U.S.A., 78, 6826-6830 (1981) and by Chandra et al., Biochem. Biophys. Res. Comm., 103, 751-758 (1981), the disclosures of which are incorp - rated herein by reference. A primate gene for alpha-1-antitrypsin may be obtained by DNA cloning methods described by Chandra et al., ibid. The gene coding for the predominant form of human alpha-1-antitrypsin, isolat d from a human cDNA library by using the baboon

sequence as a DNA hybridization probe is shown in FIG. 1A.

The human alpha-1-antitrypsin has a BamHI restriction site which allows the cutting of the gene with the removal of information for a single glutamic acid from the mature protein. Various schemes can be employed for introducing the human alpha-1-antitrypsin gene adjacent the glycolytic promoter to be under the regulation of the promoter. Where the promoter does not have a convenient restriction site near the f-met codon, the glycolytic gene may be cleaved and chewed back to the promoter with Bal31. A linker may then be introduced downstream from the promoter to provide a convenient cohesive end or flush end for joining to the human alpha-1-antitrypsin gene. The linker can also provide one or more codons for amino acids at the N-terminus of the alpha-1-antitrypsin gene, which may be the same or different from the naturally occurring amino acids.

The gene for human alpha-1-antitrypsin may then be inserted into the extrachromosomal element downstream from the glycolytic promoter, where an f-met codon is provided for initiation of expression of the human alpha-1-antitrypsin.

For example, the cDNA coding for alpha-1-antitrypsin (hereinafter "AT") may then be inserted into an expression vector, such as CTEA32 (FIG. 2), which contains the yeast promoter for triose phosphate isomerase (TPI) inserted at the BamHI site of the shuttle plasmid, CV13 [Broach J.R., Strathern J.N., Hicks J.B., Gene, 8:121-133 (1979)]. A synthetic DNA adaptor was ligated into the TPI promoter after the TPI structural sequences were removed by BAL31 dig stion from the KpnI restriction site within the TPI coding region. (Alber et al, J. Molec. Applied Genet., 1,

419-434 (1982)). This adaptor contained an ATG codon for translation initiation, followed by the sequence GAGGATCC. The GAG codon specifies a glutamic acid residue, which is the first amino acid of the naturally-occurring human AT. The GGATCC portion of the adaptor is a cutting site for BamHI endonuclease and allows for the splicing of the remainder of human AT DNA sequence into this vector.

The BamHI site of CTEA32 was constructed to be "in frame" with the rest of the AT structural gene, thereby allowing for the expression of the polypeptide when a BamHI fragment from the cloned cDNA is appropriately inserted into CTEA32. The plasmid consisting of CTEA32 plus the AT gene is called CAT1 (FIG. 2).

This DNA construct containing the gene for human AT located downstream to a yeast triose phosphate isomerase (TPI) promoter fragment was transformed into yeast strains, N501-B and GK100. Transformation into yeast is described by Beggs, Nature, 275, 104-109 (1978). Screening of the transformed yeast strains by immunological assays (competition assays and ELISA assays, using antibodies against alpha-1-antitrypsin) confirmed the presence of large amounts of human AT in yeast made from the plasmid CAT1. The "wild-type" yeast strain, N501-1B (described by Kawasaki et al., Biochem. Biophys. Res. Comm., 108, 1107-1112 (1982)), when transformed with CAT1, produced 1.8 mg alpha-1-antitrypsin per gram of soluble protein (or 0.18% alpha-1-antitrypsin), when grown at 30° on a synthetic minimal medium (modified Wickerham's medium) with 6% glucose. A mutant yeast strain, GK100, when transformed with CAT1, produced 10-15 mg alpha-1-antitrypsin per gram soluble protein (or 1-1.5% alpha-1-antitrypsin) under the same growth conditions. Strains N501-1B and GK100 each carry a defective LEU2 gene which allows for the selective

maintenance on minimal and leucine-less media of CV13 and CV13-derived plasmids (such as CAT1) which each contain a functional LEU2 gene. When grown on minimal media with only CV13 as a control, N501-1B and GK100 produce no detectable AT. Thus, AT may be specifically produced by the CAT1 plasmid.

Since GK100 produces significantly more AT than N501-1B, it is preferred. However, the present invention is not limited to AT production by GK100. It may be desirable to utilize mutations in GK100 which lead to hyperproduction of AT.

An immuno-adsorption column, made according to the method of Cuatrecasas, P. J. Biol. Chem., 245, 3059 (1970), was prepared by covalently attaching affinity-purified goat antibodies to human AT to CNBr-activated Sepharose. Disrupted GK100 yeast cells were extracted with 3 volumes of phosphate buffered saline pH 7.2 containing 0.5M NaCl, and the extracts were applied to the column. Yeast produced human AT (0.5-1.0 mg) was eluted from the column with 3M NaSCN. After the material was dialyzed to remove salt it was analyzed by electrophoresis on a polyacrylamide gel in the presence of sodium dodecyl sulfate, the results of which are shown in FIG. 3. Based on the relative migration of the protein in the gel, the approximate molecular weight of the human alpha-1-antitrypsin made in yeast is 42,000-43,000 daltons. Naturally occurring human AT has a molecular weight of approximately 54,000 daltons, having a carbohydrate composition of approximately 16% by weight, as shown by Hodges et al, J. Biol. Chem., 254, 8208-8212 (1979). It therefore appears that the yeast produced AT may be unglycosylated or substantially unglycosylated and may lack carbohydrate portions present in the naturally occurring protein.

Alternatively, other expression vectors may be constructed which contain a segment coding for alpha-1-antitrypsin. Such expression vectors may be constructed by methods known to those of ordinary skill in the art using available DNA constructs. A preferred vector is plasmid C1/1, which is more stable than CV13 and CV13 derived vectors, such as CAT1. C1/1 was constructed from plasmid, pJDB248 (Beggs, J., Nature, 275, 104-109 (1978)). The pMB9 sequences were removed from pJDB248 by partial digestion with Eco RI and were replaced by pBR322 DNA which was cut with Eco RI. The restriction map of C1/1 is given in FIG. 4. The C1/1 plasmid contains the entire 2-micron DNA from yeast (S.cerevisiae), with a pBR322 insertion at an EcoRI site. It also contains the LEU2 gene. Thus, the yeast TPI promotor with the adaptor may be inserted into the single BamHI site in the Tc^R gene of C1/1. Then the AT sequence, attached to a transcription terminator fragment from the yeast TPI gene, may be inserted into the BamHI side downstream from the TPI promotor. The resulting plasmid, HAT4, may then be transformed into N501-1B and GK100 in a manner as described above.

The sequence of the first ten amino acids in the yeast-produced AT may be confirmed by amino acid sequence analysis as identical to the first ten amino acids of the naturally occurring human AT:

$$H_2N\text{-}Glu^{1}\text{-}Asp^{2}\text{-}Pro^{3}\text{-}Gln^{4}\text{-}Gly^{5}\text{-}Asp^{6}\text{-}Ala^{7}\text{-}Ala^{8}\text{-}Gln^{9}\text{-}Lys^{10}\text{-}$$

The yeast-produced AT does not contain the initiation methionine which is specified by the ATG start codon. Therefore, the yeast cell processes off the methionine to produce the amino acid sequence of natural human AT.

The polypeptides produced according t the present invention having AT activity may be useful for treatment

of a genetic AT deficiency and other diseased states related to inadequate levels of AT. Thus, conditions such as emphysema and other lung disorders related to progressive digestion of lung sacs may be treated, such as, chronic obstructive pulmonary disease or adult respiratory distress syndrome. Non-genetically related emphysema may also be treated, such as, emphysema resulting from heavy smoking. Conditions not necessarily confined to the lungs may also be treated, such as, cystic fibrosis and arthritis. For a review of AT deficiency, see Gadek, J.E., and R. Crystal, "Alpha-1-Antitrypsin Deficiency", The Metabolic Basis of Inherited Disease, J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson, McGraw-Hill, N.Y. pp. 1450-67 (1982).

The alpha-1-antitrypsin can be used as an antigen for production of polyclonal and monoclonal antibodies to human alpha-1-antitrypsin, for introduction into a host having a deficiency of alpha-1-antitrypsin, or for modulating proteolytic activity in a mammalian host. In Partikular, the alpha-1-antitrypsin can be administered to humans to replace alpha-1-antitrypsin which has been inactivated (oxidized) by tobacco and other smoke.

The polypeptides according to the present invention may be admixed with conventional pharmaceutical carriers. Preferably, the polypeptides are to be administered intravenously or by inhalation. While the effective dosages may vary according to the severity of the condition and weight of the subject, dosages in the range of 0.5-10.0 gm/week of a polypeptide introduced intravenously may, in many cases, be effective. Lower dosages may be effective if the method of administration is by inhalation. Oral administration may also be effective provided the AT is protected in capsules or coated carriers from premature degradation in the digestive tract.

The following examples set forth specific embodiments according to the present invention, but the invention is not intended to be limited thereto.

EXAMPLE 1

Strains. Isogenic strains carrying mutations in PGI1, PGK1, GPM1, PYK1, and GCR1 where obtained by ethyl methane sulfonate (EMS) mutagenesis of S. cerevisiae (S. c.) X2180-1A (MATa SUC2 CUP1 gal2, from the Berkeley Yeast Stock Center). 35,000 independent colonies were grown on YEP-3% glycerol-2% ethanol and were screened by replica plating for the inability to grow on YEP-4% dextrose (Table 1).

Identification of specific lesions was made by complementation tests with known glycolysis mutants (Ciriacy and Breitenbach, J.Bacteriol, 139:152-60 (1979)), while at least 15 additional complementation groups were found by intercrossing mutant strains. Enzyme assays (Clifton et al. Genetics, 88:1-11 (1980)) confirmed the glycolytic defects in pgi1, pgk1, gpm1, pyk1, and gcr1 mutants.

A LEU2 mutant was also derived from S.cerevisiae X2180-1A by EMS treatment and was crossed to X2180-1B (an isogenic MATα strain) to produce N501-1B (MATα leu2 SUC2 CUP1 gal2). Cycloheximide (cyh2) and canavanine (can1) resistances were then selected as spontaneous mutations in N501-1B. The glycolysis mutants were crossed to N501-1B to produce a series of isogenic leu2 strains each defective in a single glycolytic function or in GCR1.

A tpi1 mutant, S. cerevisiae GLU77 was crossed to N551-1A (MATa leu2 SUC2 CUP1 gal2); strains derived from this mating were crossed twice to N501-1B to produce a

tpi1 leu2 strain, N587-2D, which was similar in genetic background to the other Glycolysis mutants.

Mutations in three glucose phosphorylating enzymes produce a strain which is unable to grow on dextrose as the sole carbon source and which is resistant to catabolite repression by 2-deoxyglucose and glucosamine. N517-6C (hxk1 hxk2 glk1 leu2 can1-100 cyh2 ade2-1) was derived from a hxk1 hxk2 glk1 strains, D308.3, by screening for glucosamine- resistant spore colonies. Defects in glucose kinasing activities were confirmed by assay.

TABLE I

Complementation Groups of *glu*⁻ Derivatives of X2180-1A

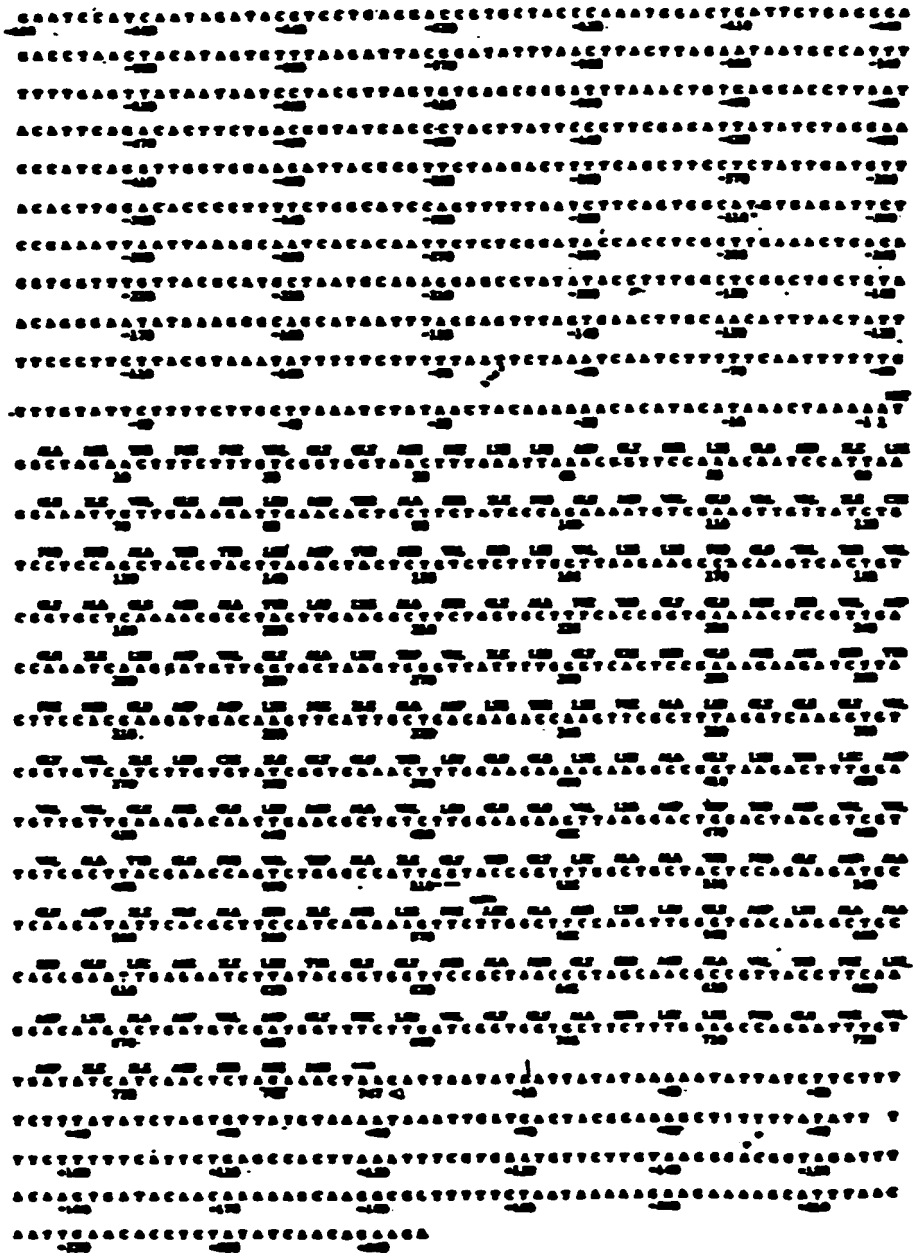
Gene	No. of Mutants	
PYK1	14	
PDC1	9	
GCR1	4	
PGI1	3	
GPM1	3	
PGK1	1	
TPI1	0	
FDP	0	
(LEU2)	(1)	
I	11	
II	10	
III	3	
IV	5	
V	1	
VI	1	
VII	2	
VIII	3	60 other mutations not in
IX	2	the complementation groups
X	3	
XI	2	
XII	1	
XIII	1	
XIV	5	
XV	1	

27 sterile *glu*⁻ strains

35,000 colonies screened (EMS mutagenized for 50% kill)

The homothallic diploid strain, S. c. AB320 was the source of the yeast DNA pool (Nasmyth and Reed, Proc. Nat. Acad. Sci., 77:2119-2123 (1980) and was used as a control in some experiments.

The triose phosphate isomerase gene (including the upstream sequence having the regulatory signals) is as follows:



The pyruvate kinase gene upstream sequenc having the regulatory signals is as follows:

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        10          20          30          40          50          60
GAATTCAGCA TCATAGCTAC GTAAAATGTGT TCCGCACCGT CACAAAGTGT TTTCTACTGT
CTTAAGTCGT AGTATCGATG CATTTACACA AGGCGTGGCA GTGTTTCACA AAACATGACA

        70          80          90         100         110         120
TCTTTCTTCT TTCGTTCATT CAGTTCAGTT GACTGAGTGC TTTGTTCAAT CGATCTTAGC
AGAAAGAAGA AAGCAAGTAA GTCAATTCAA CTCACTCACG AAACAAGTTA GCTAGAATCG

        130         140         150         160         170         180
TAAAATGCAT ATTTTTTCTC TTGGTAAATG AATGCTTGTG ATGTCTTCCA AGTGATTTCC
ATTTTACGTA TAAAAAAGAG AACCATTTAC TTACGAACAC TACAGAAGGT TCACTAAAGG

        190         200         210         220         230         240
TTTCCTTCCC ATATGATGCT AGGTACCTTT AGTGTCTTCC TAAAAAAAAA AAAAGGCTCG
AAAGGAAGGG TATACTACGA TCCATGGAAA TCACAGAAGG ATTTTTTTTT TTTTCCGAGC

        250         260         270         280         290         300
CCATCAAAAC GATATTCGTT GGCTTTTTTT TCTGAATTAT AAATACTCTT TGGTAACTTT
GGTAGTTTTG CTATAAGCAA CCGAAAAAAA AGACTTAATA TTTATGAGAA ACCATTGAAA

        310         320         330         340         350         360
TCATTTCCAA GAACCTCTTT TTTCCAGTTA TATCATGGTC CCCTTTCAAA GTTATTCTCT
AGTAAAGGTT CTTGGAGAAA AAAGGTCAAT ATAGTACCAG GGGAAAGTTT CAATAAGAGA

        370         380         390         400         410         420
ACTCTTTTTC ATATTCATTC TTTTTCATCC TTTGGTTTTT TATTCTTAAC TTGTTTATTA
TGAGAAAAAG TATAAGTAAG AAAAAGTAGG AAACCAAAAA ATAAGAATTG AACAAATAAT

        430         440         450         460         470         480
TTCTCTCTTG TTTCTATTTA CAAGACACCA ATCAAAACAA ATAAAACATC ATCACAATGT
AAGAGAGAAC AAAGATAAAT GTTCTGTGGT TAGTTTTGTT TATTTTGTAG TAGTGTTACA

        490         500         510         520         530         540
CTAGATTAGA AAGATTGACC TCATTAAACG TTGTTGCTGG TTCTGACTTG AGAAGAACCT
GATCTAATCT TTCTAACTGG AGTAATTTGC AACAACGACC AAGACTGAAC TCTTCTTGGA

        550         560         570         580         590         600
CCATCATTGG TACCATCGGT TCAAAGACCA ACAACCCAGA AACCTTGGTT GCTTTGAGAA
GGTAGTAACC ATGGTAGCCA AGTTTCTGGT TGTTGGGTCT TTGGAACCAA CGAAACTCTT

        610         620         630         640         650         660
AGGCTGGTTT GAACATTGTT CGTATGAACT TCTCTCACGG TTCTTACGAA TACCACAAGT
TCCGACCAAA CTTGTAACAA GCATACTTGA AGAGAGTGCC AAGAATGCTT ATGGTGTTCA

        670         680         690         700
CTGTCGTTGA CAACGCCACA AAGTCCGAAG AATTGTACCC
GACAGCAACT GTTGCGGTGT TTCAGGCTTC TTAACATGGG
```

Screening of clone bank. The leu2 glycolysis mutants were transformed with a yeast DNA pool inserted into pYE13, a high copy plasmid carrying a selectable LEU2 wild-type gene (Broach et al., Gene, 8:121-133 (1979)). The glycolytic genes were obtained by complementation, involving the simultaneous selection for growth on glucose and leucine prototrophy. A synthetic medium containing yeast nitrogen base, 4% glucose, and the following supplements was used: per liter, 40mg adenine, 20mg arginine, 50mg aspartate, 10mg histidine, 60mg isoleucine, 40mg lysine, 10mg methionine, 60mg phenylalanine, 50mg threonine, 40mg tryptophan, 50mg tyrosine, 20mg uracil, and 60mg valine.

The transformants were purified on leucineless media and were then grown on a non-selective medium (YEPGE) to allow mitotic segregation of the plasmids. Strains which cosegregated the leu2 and glycolysis mutant phenotypes, as determined by replica plating on selective media, were assayed for glycolytic enzyme activities. Yeast DNA preps were made, and the E. coli strain, RR1, was transformed, selecting for ampicillin resistance, to verify the presence of plasmid DNAs in these yeast glycolytic transformants.

Enzyme Assays. The transformed yeast strains were selectively grown on minimal medium with 8% glucose (adenine was added to a final concentration of 50mg/l). The wild-type control, N501-1B, was grown on the same medium plus leucine (100mg/l). The glycolysis mutant strains were grown on YEP-5% glycerol-1% lactate. Overnight cultures were fed fresh media and were aerobically grown at 30° for four hours before harvesting. The cells were washed two times with water and resuspended in 50mM K_2HPO_4 2mM EDTA 3mM 2-mercaptoethanol (adjusted to pH7.4 with HCl). Extracts wer obtained by vortexing the cells with an equal volume of

glass beads (.45 mm diam.) at high speed for two minutes. The cell debris was removed by centrifugation in a microfuge for 15 min. at 4°. Enzymes were assayed as described by Clifton and Breitenbach, supra. Protein concentrations were determined by the Biuret-TCA method.

EXAMPLE 2

In order to determine the activity of the various glycolytic genes in the transformants, the various enzymes were assayed and the results for the transformants were compared to mutant and wild-type strains. The gcr1 mutant had 5-10% of the wild-type levels of most glycolytic activities (exemplified by PGI, aldolase and enolase) and grows very poorly on glucose media. In contrast, the GCR1 transformants had nearly wild-type levels of enzymes and were virtually identical to wild-type for growth on glucose media. The other glycolysis mutants had less than 5% of the normal levels of their respective enzyme activities. However, when transformed with a complementing high copy plasmid, the specific enzyme activities were substantially elevated above wild-type levels (typically 5-10 fold higher). The following Table 2 indicates the results.

TABLE 2

Comparison of Glycolytic Activities in Wild-type, Mutant, and Transformed Strains

Enzyme	Activities			
	Wild-type[a]	Mutant[b]	Transformant[c]	Ratio: Transf/Wt
PGI	2.85	.0065	31.49 (10)	11.1
TPI	18.3	.0000	167.8 (10)	9.2
PGK	1.99	.0046	17.67 (3)	8.9
GPM	0.74	.0000	4.80 (10)	6.5
PYK	4.02	.0057	14.77 (10)	3.7
	Wild-type[a]	gcr1 Mutant[d]	GCR1 Transf[c]	
PGI	2.85	.2436	2.42 (10)	.85
Aldolase	4.33	.4415	2.96 (10)	.68
Enolase	0.43	.0274	.316 (10)	.74

[a]Wild-type is N501-1B.

[b]The respective mutant strains are N543-9D (pgi1 leu2), N587-2D (tpi1 leu2), N548-8A (pkg1 leu2), N583-2C (gpm1 leu2), and N549-3A (pyk1 leu2).

[c]The activities of the transformants are averages for many different isolates. The numbers in parentheses represent the numbers of independent transformants assayed.

[d]The gcr1 leu2 mutant strain is N525-2C.

EXAMPLE 3

In order to demonstrate that the hyperproduction of glycolytic enzymes was specific to the mutational defect complemented by the particular plasmid, assays for ten

different glycolytic proteins were conducted on the various transformants. The following Table 3 reports the results for one transformant for each of the six different glycolysis genes which were examined in detail.

TABLE 3

RELATIVE ENZYME ACTIVITIES OF WILD-TYPE AND TRANSFORMED GLYCOLYTIC ENZYMES

Strains	GLK	PGI	PFK	FBA	TPI	GLD	PG
N501-1B	1.00	1.00	1.00	1.00	1.00	1.00	1.0
Transformant GCR-8	1.05	0.63	1.44	0.79	0.62	0.63	0.7
Transformant PGI-19	0.64	5.63	1.26	0.57	0.58	0.75	0.5
Transf rmant TPI-10	0.99	0.77	1.35	0.99	13.85	0.87	0.6
Transformant PGK-2	0,54	0.45	1.05	0.54	0.46	0.63	2.9
Transformant GPM-2	0.97	0.82	1.69	1.02	1.02	0.85	0.9
Transformant PYK-1	1.02	0.83	1.09	0.89	1.22	0.84	1.2

The GCR-8 transformant gave nearly wild-type levels of all ten enzymes, while PGI-19, TPI-10, PGK-2, GPM-2 and PYK-1 transformants overproduced their respective glycolytic proteins, but not other enzymes.

It was noted that the plasmids readily segregated (typically 5-50% segregation in fully grown cultures even under selective pressure of leucine prototrophy, so the assayed cultures probably contain cells with a range of number of plasmids. By complementation in E. coli and/or sequencing, TPI1 and PYK1 have both been shown to be the structural gene.

EXAMPLE 4

Exploitation of the promoter for TPI1 for the production of human alpha-1-antitrypsin was demonstrated as follows. The plasmid CV13 was employed. CV13 can be maintained by selection of yeast with an average of about ten copies per cell. CV13 is comprised of pBR322, the replicon for the 2μ-plasmid and the yeast LEU2 gene. TPI1 promoter fragment was obtained by cutting the TPI1 gene at the unique KpnI site (bases 511 to 518); and the resulting linearized DNA was then treated with Bal31 for four to five minutes in order to remove the TPI1 structural sequences. Linkers, either EcoRI, Hind III or BamHI, were then inserted. The linkers will then cleave with the appropriate Restriction enzyme to provide cohesive ends for insertion of human alpha-1-antitrypsin genes. The human alpha-1-antitrypsin gene was digested with BamHI, which cleaves at the 5'-terminus of the coding strand to remove the information for a single glutamic acid codon from the mature protein. Four different constructions were prepared, as set forth in the following Table 4. From this table it is noted that the glutamic acid codon is substituted by the codons for alanine and proline in three of the constructions having the initiator methionine.

After ligation of the human alpha-1-antitrypsin construction into the CV13 plasmid, the resulting plasmid was transformed into S. c. N501-1B. The resulting yeast cells were then grown on a minimal synthetic medium.

TABLE 4

Plasmid	N-terminal amino acid	Orientation in CV13
CAT1	met glu + hAT*	clockwise
C-Tα2	met ala pro + hAT	counterclockwise
C-Tα1	met ala pro + hAT	clockwise
C-TSα2	met ala pro + hAT, but missing part of TPI promoter	counterclockwise

*remainder of approximately 400 amino acids of human alpha-1-antitrypsin

Yeast cells containing the human alpha-1-antitrypsin genes were broken open by vortexing with glass beads (0.45mm) at high speed for 2-3 minutes. The extraction buffer contained 50mM K_2HPO_4, 2mM EDTA, 2mM 2-mercaptoethanol and 1mM PMSF (pH7.4) cell debris was removed by centrifugation and the extracts contain 3-4mg/ml protein as determined by Lowry assays.

The presence of human alpha-1-antitrypsin was determined using a RIA, employing tritium-labeled human alpha-1-antitrypsin and antibody directed against the protein. The following Table 5 indicates the results.

TABLE 5

Competition assay for alpha-1 antitrypsin

Plasmid	Tritium Counts	Average Count	α-1-AT [µg]	Total Protein (µg)	%Total Protein
CAT1	46010 52257	49133.5	0.75	420	.18
C-Tα2	12268 13330	12799	3.35	380	.88
C+Tα1	41635 39071	40353	0.95	360	.26
C-TSα2	66490 70038	68264	0	345	0

Controls**	Counts
0 µg α-1	68440
0.25 µg α-1	65333
0.5 µg α-1	58928
1.0 µg α-1	38468
2.0 µg α-1	19559
3.0 µg α-1	14432
4.0 µg α-1	11155
5.0 µg α-1	9615

*Plasmids were grown in yeast strain, N501-1B. 100µl of extracts were assayed.

**Non-radioactive alpha-1-antitypsin mixed with 100µl of yeast extract (330µg protein)

It is evident from the above results that an immunologically active product is obtained, which is capable of competing with naturally occurring human alpha-1-antitrypsin for antibodies to the native protein.

Furthermore, the expression of the alpha-1-antitrypsin gene is regulated by the TPI promoter, for as is seen, where a portion of the TPI promoter is removed, no alpha-1-antitrypsin is produced. In addition, the production of the mammalian protein human alpha-1-antitrypsin has not been optimized in the above study, so that the results indicate a minimum production of product which can be further enhanced. Thus, the TPI promoter is found to be an effective promoter for efficiently producing high yields of expression products of alien DNA.

EXAMPLE 5

Purification Of Alpha-1-Antitrypsin From Yeast GK100 Yeast Extracts

An immuno adsorption column was prepared by covalently attaching affinity-purified antibodies to human alpha-1-antitrypsin to CNBr-activated Sepharose according to the method of Cuatrecasas, J. Biol. Chem., 245, 3059 (1970). Disrupted GK100 cells were extracted with three volumes of phosphate buffered saline pH 7.2 containing 0.5M NaCl and applied to the column. The column was eluted with 3M NaSCN and the recovered material was analyzed by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate. The results of the electrophoresis are shown in FIG. 3. Track 1 contained a mixture of molecular weight standards: a) phosphorylase B, 97,000 daltons; b) bovine serum albumen (BSA), 65,000 daltons; c) ovalbumin, 43,500 daltons; d) carbonic anhydrase, 30,000 daltons; e) soybean trypsin inhibitor, 20,000 daltons; and f) alpha-lactalbumin, 14,000 daltons. Track 3 contains yeast produced AT purified by immunoadsorption, molecular weight about 42,000 daltons. Track 7 is a sample of naturally occurring AT purchased from Sigma Chemical Company, heavily contaminated by

blood proteins. A major component of Track 7 is human alpha-1-antitrypsin, molecular weight 54,000 daltons.

EXAMPLE 6

Activity Of Yeast Produced Alpha-1-Antitrypsin Against Serine Protease Trypsin

As a control, 10 microliters (1 microgram) of a solution of 100 microgram/ml trypsin, 100 microgram (100 microliters) of bovine serum albumin and 100 microliters of 0.05 molar TRIS, pH 8.0 buffer containing 1mM benzoyl-arginioyl-p-nitroanilide were mixed, and the increase in absorbance at 405 nm was measured over time in a spectrophotometer. The absorbance value of this solution was used as a standard for 100% trypsin activity. Three additional samples were run in duplicate, each containing 1 µl trypsin and, respectively containing 25 µl AT solution plus 175 µl buffer, 100 µl alpha-1-antitrypsin plus 100 µl buffer, and 200 µl alpha-1-antitrypsin. All samples contain equal concentrations of substrate and bovine serum albumin. The results demonstrated that utilizing 25 microliters of AT, 73% of the trypsin activity remained, with 100 microliters of AT, 41% of trypsin activity remained and with 200 microliters of alpha-1-antitrypsin, 26% of the trypsin activity remained. This demonstrated that by increasing the levels of the yeast made AT the trypsin inhibitory activity also increased.

EXAMPLE 7

Production of Alpha-1-Antitrypsin From Yeast Plasmids With Increased Genetic Stability

Increased levels of AT may be obtained by utilizing C1/1, a plasmid which is more genetically stable than

CV13. The C1/1 plasmid contains the entire 2-micron DNA from S. cerevisiae and, therefore, can promote its own replication and maintenance in yeast in the absence of selection for a genetic marker. Also, C1/1 plasmid has a single BamHI site located in the Tc^R gene. Transformants carrying C1/1 may be selected in E. coli by ampicillin- or tetracycline-resistance and in yeast by leucine prototrophy. C1/1 contains pBR322 inserted into an EcoRI site of 2-micron DNA and carries the LEU2 gene described by J. Beggs, Nature, 275, 104-109 (1978).

The yeast TPI promoter (from CTEA32) with the synthetic DNA adaptor (described above) was inserted as a Bgl II - BamHI fragment (about 900 base pairs) into the BamHI site of C1/1. This insertion created a single BamHI site into which the human AT gene could be spliced for expression in yeast. As in the CAT1 plasmid, when the AT gene (FIG. 1A) is inserted, the resultant plasmid would have an ATG initiation codon followed by a GAG (glutamic acid codon) to allow the production of mature human AT protein sequence in yeast.

About 700 base pairs of the 3' flanking region of th yeast TPI gene was added after the human AT sequence to assist in transcription termination. The "termination" fragments are sequences from the XbaI to EcoRI sites in the plasmid TPIC10 (T. Alber and G. Kawasaki, J. Molec. Applied Genet., 1, 419-434 (1982)).

The yeast termination sequences were attached to the human AT gene by using the vector, pUC13, which has multiple cloning sites into which the terminator and AT DNA's can be separately inserted. The pUC13 plasmid was constructed as described in Vieira, J., and Messing, J., Gene, 19, 259-268 (1982) for vectors, pUC8 and pUC9. The pUC13 plasmid contained the multiple restriction

site, depicted in FIG. 5, at the start of the lac Z gene. To connect the human AT gene to the TPI transcription terminator, the AT cDNA clone (FIG. 1) was inserted as a Pst I fragment into pUC13 at the single Pst I site. The AT gene was followed by an Xba I site and Eco RI site in the multiple cloning sequence. Between these Xba I and Eco RI sites of pUC13 was inserted the yeast TPI terminator as a 700 base pair Xba I-Eco RI fragment from pTPIC10. The resulting plasmid, pUCa1+FG1, contained a human AT gene with a yeast transcription terminator (See FIG. 6). An Eco RI-Bam HI synthetic DNA adaptor was then added to the Eco RI site of the plasmid, in order to create a Bam HI site on the 5' end of the yeast terminator. By using this adaptor, the human AT-yeast terminator sequence could be removed by cutting with Bam HI to liberate a fragment of approximately 2100 base pairs. This BamHI fragment was inserted into the C1/1 plasmid containing the TPI promoter with BamHI adaptor. The resulting plasmid, HAT4, has the TPI promoter, ATGGAGGATCC adapter, human AT gene (from the BamHI site), and TPI terminator inserted into C1/1. The topology of HAT4 is depicted in FIG. 7.

HAT4 was transformed into N501-1B and GK100. On minimal media with 6% glucose, 2-3% of the yeast soluble protein was alpha-1-antitrypsin at a cell density of nearly 3g per liter (wet weight). Because HAT4 contained C1/1, this plasmid was maintainable in a variety of rich media, including YEPD (1% yeast extract, 2% peptone, and 2% glucose). On rich media 2-3% AT was still produced but at a higher cell density of 10-20g per liter (wet weight). The HAT4 plasmid was maintained without selection in N501-1B for over 30 divisions on rich media with greater than 70% of the cells containing the

plasmid. In GK100 better than 95% of the cells had HAT4 after 30 divisions on rich media. The advantages of using HAT4 over CAT1 were 1) greater plasmid stability, 2) higher levels of AT as a percentage of total protein, 3) much greater yields of cells per liter as a result of using rich media, and 4) cheaper costs of rich media compared to synthetic (leucine-less) media. The mutant yeast strain GK100 has been placed on deposit in the American Type Culture Collection, Rockville, Maryland, ATCC No. 20669.

It is evident from the above results that yeast promoters can be efficiently used for the production of foreign proteins by regulating the expression of alien DNA in yeast. The promoters are found to be strong promoters, so as to provide for a high degree of expression. Furthermore, it would appear that the messengers are sufficiently stable as to allow for a significant degree of translation into the desired expression product. Furthermore, by employing the glycolytic promoters and appropriate nutrient media, the expression of the alien DNA can be modulated. In this way, production of the alien DNA can be turned on and off. Thus, the subject invention provides a method for using yeast as efficient host in the production of foreign proteins, where the production may be modulated. In addition, by using the glycolytic regulation gene, one can turn on and off a plurality of glycolytic promoters.

CLAIMS:

1. An extrachromosomal element capable of replication in yeast and containing a yeast promoter capable of regulating the transcription of a glycolytic protein, which protein is triose phosphate isomerase, pyruvate kinase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructose kinase, and aldolase, or glycolytic regulation protein, said yeast promoter being followed downstream by other than a gene expressing the protein normally regulated by such promoter.

2. An extrachromosomal element according to Claim 1, having a marker for selection in a yeast host.

3. An extrachromosomal element according to Claim 1, having a gene expressing a foreign protein downstream from said promoter and under the regulation of said promoter.

4. An extrachromosomal element according to Claim 1, wherein said promoter is followed by a gene expressing protease inhibitor having substantially the same structure as alpha-1-antitrypsin.

5. An extrachromosomal element according to any of Claims 1, 2, 3 or 4, wherein said promoter is the triose phosphate isomerase promoter or the pyruvate kinase promoter.

6. An extrachromosomal element according to Claim 5, wherein a gene expressing an enzyme in a metabolic path way of said yeast is present.

7. A yeast cell containing an extrachromosomal element according to any of Claims 1, 2, 3 or 4.

8. A DNA construct comprising a triose phosphate isomerase promoter followed by a gene expressing a foreign protein under the regulation of said promoter.

9. A DNA construct according to claim 8, wherein said foreign protein is a protein having protease inhibition activity.

10. A DNA construct according to Claim 9, wherein the major sequence of said protein is the same sequence amino acids as human alpha-1-antitrypsin.

11. A DNA construct comprising a pyruvate kinase promoter followed by a gene expressing a foreign protein under regulation of said protein.

12. A method for preparing any foreign protein which comprises: introducing into a yeast host an extrachromosomal element according to Claims 1, 2, 3, or 4; and growing said yeast host in an appropriate medium and isolating the protein expressed by said foreign DNA.

13. A yeast cell containing at least a portion of an extrachromosomal element according to any of Claims 1, 2, 3 or 4 integrated into the genome of said yeast cell, wherein said portion includes at least said promoter and said gene.

14. A yeast cell according to Claim 13, wherein said promoter is the triose phosphate isomerase promoter

15. A protease inhibitor produced by culturing a yeast cell having an extrachromosomal element according to Claim 4.

16. A protease inhibitor produced by culturing a yeast cell containing at least a portion of an extrachromosomal element integrated into said yeast cell genome, wherein said portion includes a yeast promoter capable of regulating the transcription of a glycolytic protein, which protein is triose phosphate isomerase, pyruvate kinase, phosphoglucose isomerase, phosphoglycerate mutase, hexokinase 1, hexokinase 2, glucokinase, phosphofructose kinase, and aldolase, or glycolytic regulation protein, and a gene under the transcription regulation of said promoter and expressing a protease inhibitor having substantially the same polypeptide structure as alpha-1-antitrypsin.

17. A method of producing a polypeptide having the protease inhibition activity of mammalian alpha-1-antitrypsin, comprising the step of growing a culture of microorganisms transformed by a DNA transfer vector, said vector comprising a segment coding for mammalian alpha-1-antitrypsin.

18. A method according to Claim 17 wherein said culture comprises fungal microorganisms.

19. A method according to Claim 18 wherein said microorganisms comprise yeast cells.

20. A method according to Claim 19 wherein said yeast cells comprise cells of the mutant strain GK-100.

21. A method according to Claim 19 wherein said yeast cells contain allelic mutations to GK-100 mutations whereby hyperproduction of said polypeptide is attained.

22. A method according to Claim 19 wherein said vector comprises 2-micron plasmid DNA, plasmid CATI or plasmid HAT4.

23. A method according to Claim 17 further comprising the steps of extracting said polypeptide from said culture and purifying said polypeptide.

24. A method according to Claim 23 further comprising the step of altering a suitable vector to include said segment coding for mammalian alpha-1-antitrypson to form said DNA transfer vector.

25. A method according to Claim 23 further comprising the step of introducing said DNA transfer vector into yeast.

26. A method according to any of Claims 20 to 30 wherein said polypeptide comprises the amino acid sequence of naturally-occurring mammalian alpha-1-antitrypsin.

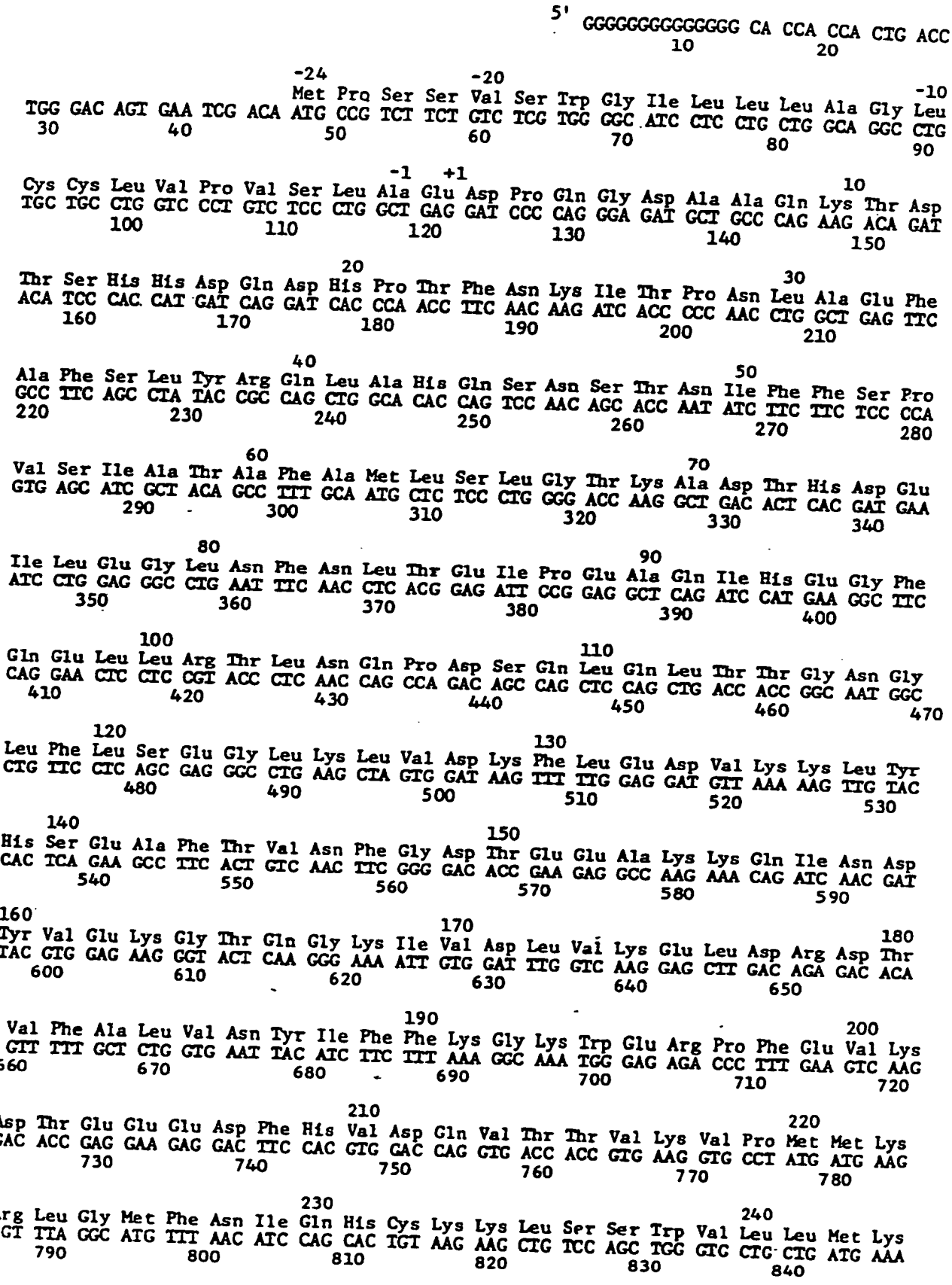
27. A method according to Claim 31 wherein said polypeptide is the predominant human form of alpha-1-antitrypsin.

28. A substantially unglycosylated polypeptide characterized by the amino acid sequence of mammalian alpha-1-antitrypsin.

29. Substantially pure, substantially unglycosylated mammalian alpha-1-antitrypsin.

30. A substantially unglycosylated polypeptide characterized by the amino acid sequence of mammalian alpha-1-antitrypsin for use in the treatment of diseased states.

FIG. 1A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TGC TGC CTG GTC CCT GTC TCC CTG GCT GAG GAT CCC CAG GGA GAT GCT GCC CAG AAG ACA GAT
100 110 120 130 140 150
20 30
Thr Ser His His Asp Gln Asp His Pro Thr Phe Asn Lys Ile Thr Pro Asn Leu Ala Glu Phe
ACA TCC CAC CAT GAT CAG GAT CAC CCA ACC TTC AAC AAG ATC ACC CCC AAC CTG GCT GAG TTC
160 170 180 190 200 210
40 50
Ala Phe Ser Leu Tyr Arg Gln Leu Ala His Gln Ser Asn Ser Thr Asn Ile Phe Phe Ser Pro
GCC TTC AGC CTA TAC CGC CAG CTG GCA CAC CAG TCC AAC AGC ACC AAT ATC TTC TTC TCC CCA
220 230 240 250 260 270 280
60 70
Val Ser Ile Ala Thr Ala Phe Ala Met Leu Ser Leu Gly Thr Lys Ala Asp Thr His Asp Glu
GTG AGC ATC GCT ACA GCC TTT GCA ATG CTC TCC CTG GGG ACC AAG GCT GAC ACT CAC GAT GAA
290 300 310 320 330 340
80 90
Ile Leu Glu Gly Leu Asn Phe Asn Leu Thr Glu Ile Pro Glu Ala Gln Ile His Glu Gly Phe
ATC CTG GAG GGC CTG AAT TTC AAC CTC ACG GAG ATT CCG GAG GCT CAG ATC CAT GAA GGC TTC
350 360 370 380 390 400
100 110
Gln Glu Leu Leu Arg Thr Leu Asn Gln Pro Asp Ser Gln Leu Gln Leu Thr Thr Gly Asn Gly
CAG GAA CTC CTC CGT ACC CTC AAC CAG CCA GAC AGC CAG CTC CAG CTG ACC ACC GGC AAT GGC
410 420 430 440 450 460 470
120 130
Leu Phe Leu Ser Glu Gly Leu Lys Leu Val Asp Lys Phe Leu Glu Asp Val Lys Lys Leu Tyr
CTG TTC CTC AGC GAG GGC CTG AAG CTA GTG GAT AAG TTT TTG GAG GAT GTT AAA AAG TTG TAC
480 490 500 510 520 530
140 150
His Ser Glu Ala Phe Thr Val Asn Phe Gly Asp Thr Glu Glu Ala Lys Lys Gln Ile Asn Asp
CAC TCA GAA GCC TTC ACT GTC AAC TTC GGG GAC ACC GAA GAG GCC AAG AAA CAG ATC AAC GAT
540 550 560 570 580 590
160 170 180
Tyr Val Glu Lys Gly Thr Gln Gly Lys Ile Val Asp Leu Val Lys Glu Leu Asp Arg Asp Thr
TAC GTG GAG AAG GGT ACT CAA GGG AAA ATT GTG GAT TTG GTC AAG GAG CTT GAC AGA GAC ACA
600 610 620 630 640 650
190 200
Val Phe Ala Leu Val Asn Tyr Ile Phe Phe Lys Gly Lys Trp Glu Arg Pro Phe Glu Val Lys
GTT TTT GCT CTG GTG AAT TAC ATC TTC TTT AAA GGC AAA TGG GAG AGA CCC TTT GAA GTC AAG
660 670 680 690 700 710 720
210 220
Asp Thr Glu Glu Glu Asp Phe His Val Asp Gln Val Thr Thr Val Lys Val Pro Met Met Lys
GAC ACC GAG GAA GAG GAC TTC CAC GTG GAC CAG GTG ACC ACC GTG AAG GTG CCT ATG ATG AAG
730 740 750 760 770 780
230 240
Arg Leu Gly Met Phe Asn Ile Gln His Cys Lys Lys Leu Ser Ser Trp Val Leu Leu Met Lys
CGT TTA GGC ATG TTT AAC ATC CAG CAC TGT AAG AAG CTG TCC AGC TGG GTC CTG CTG ATG AAA
790 800 810 820 830 840

FIG. 1A - page 2

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                                  250                                                 260
Tyr Leu Gly Asn Ala Thr Ala Ile Phe Phe Leu Pro Asp Glu Gly Lys Leu Gln His Leu Glu
TAC CTG GGC AAT GCC ACC GCC ATC TTC TTC CTG CCT GAT GAG GGG AAA CTA CAG CAC CTG GAA
850          860          870          880          890          900          910

                        270                                                 280
Asn Glu Leu Thr His Asp Ile Ile Thr Lys Phe Leu Glu Asn Glu Asp Arg Arg Ser Ala Ser
AAT GAA CTC ACC CAC GAT ATC ATC ACC AAG TTC CTG GAA AAT GAA GAC AGA AGG TCT GCC AGC
          920          930          940          950          960          970

                  290                                                 300
Leu His Leu Pro Lys Leu Ser Ile Thr Gly Thr Tyr Asp Leu Lys Ser Val Leu Gly Gln Leu
TTA CAT TTA CCC AAA CTG TCC ATT ACT GGA ACC TAT GAT CTG AAG AGC GTC CTG GGT CAA CTG
      980          990          1000         1010         1020         1030

            310                                                 320
Gly Ile Thr Lys Val Phe Ser Asn Gly Ala Asp Leu Ser Gly Val Thr Glu Glu Ala Pro Leu
GGC ATC ACT AAG GTC TTC AGC AAT GGG GCT GAC CTC TCC GGG GTC ACA GAG GAG GCA CCC CTG
 1040         1050         1060         1070         1080         1090         1100

      330                                                 340                         350
Lys Leu Ser Lys Ala Val His Lys Ala Val Leu Thr Ile Asp Glu Lys Gly Thr Glu Ala Ala
AAG CTC TCC AAG GCC GTG CAT AAG GCT GTG CTG ACC ATC GAC GAG AAA GGG ACT GAA GCT GCT
          1110         1120         1130         1140         1150         1160

                                    360                                 370
Gly Ala Met Phe Leu Glu Ala Ile Pro Met Ser Ile Pro Pro Glu Val Lys Phe Asn Lys Pro
GGG GCC ATG TTT TTA GAG GCC ATA CCC ATG TCT ATC CCC CCC GAG GTC AAG TTC AAC AAA CCC
      1170         1180         1190         1200         1210         1220

                                    380                                             390
Phe Val Phe Leu Met Ile Glu Gln Asn Thr Lys Ser Pro Leu Phe Met Gly Lys Val Val Asn
TTT GTC TTC TTA ATG ATT GAA CAA AAT ACC AAG TCT CCC CTC TTC ATG GGA AAA GTG GTG AAT
  1230         1240         1250         1260         1270         1280

            394
  Pro Thr Gln Lys STOP
  CCC ACC CAA AAA TAA CTG CCT CTC GCT CCT CAA CCC CTC CCC TCC ATC CCT GGC CCC CTC CCT
1290         1300         1310         1320         1330         1340         1350

GGA TGA CAT TAA AGA AGG GTT GAG CTG
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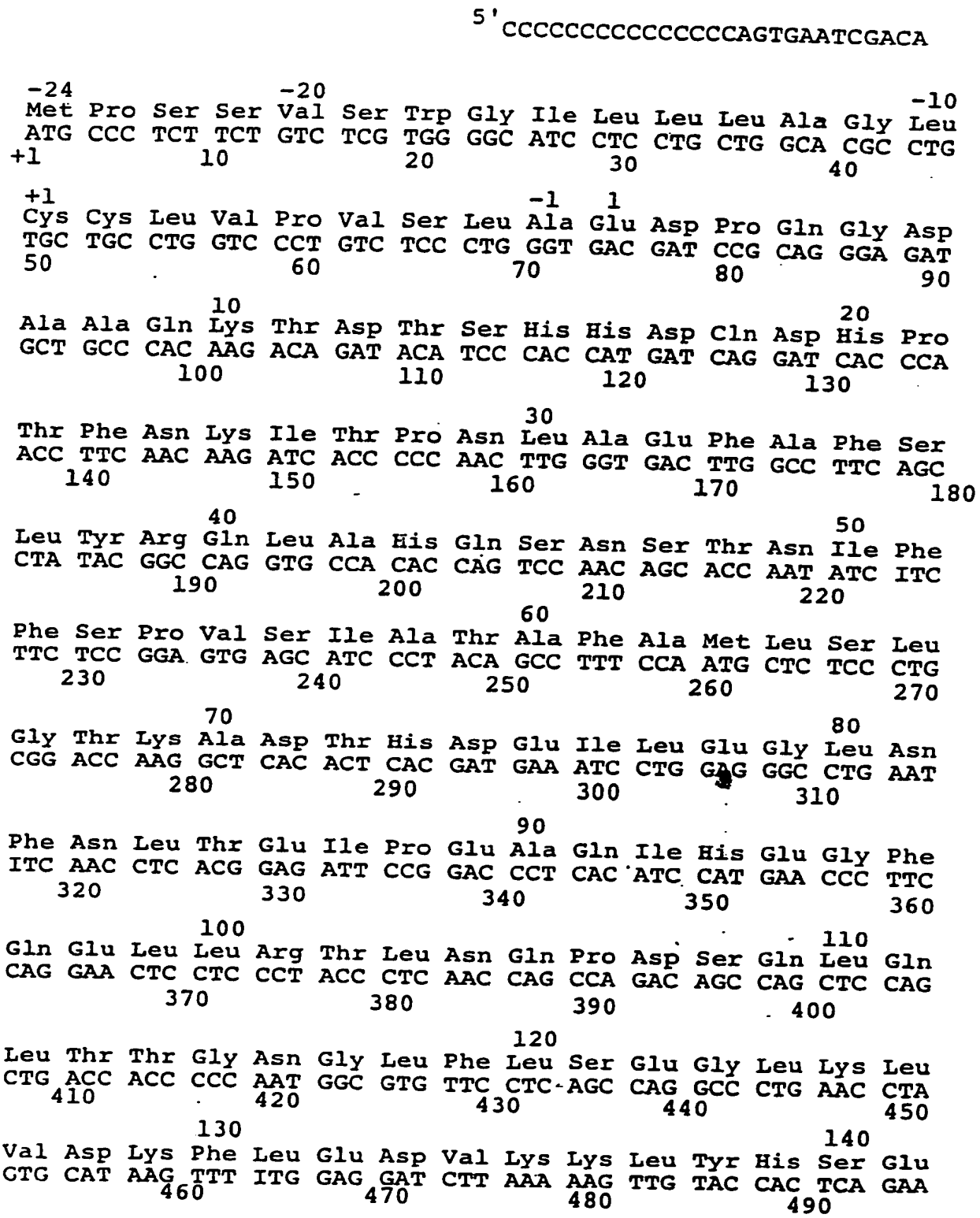
FIG. 1B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-24 -20 -10
Met Pro Ser Ser Val Ser Trp Gly Ile Leu Leu Leu Ala Gly Leu
ATG CCC TCT TCT GTC TCG TGG GGC ATC CTC CTG CTG GCA CGC CTG
+1 10 20 30 40
+1 -1 1
Cys Cys Leu Val Pro Val Ser Leu Ala Glu Asp Pro Gln Gly Asp
TGC TGC CTG GTC CCT GTC TCC CTG GGT GAC GAT CCG CAG GGA GAT
50 60 70 80 90
10 20
Ala Ala Gln Lys Thr Asp Thr Ser His His Asp Cln Asp His Pro
GCT GCC CAC AAG ACA GAT ACA TCC CAC CAT GAT CAG GAT CAC CCA
100 110 120 130
30
Thr Phe Asn Lys Ile Thr Pro Asn Leu Ala Glu Phe Ala Phe Ser
ACC TTC AAC AAG ATC ACC CCC AAC TTG GGT GAC TTG GCC TTC AGC
140 150 160 170 180
40 50
Leu Tyr Arg Gln Leu Ala His Gln Ser Asn Ser Thr Asn Ile Phe
CTA TAC GGC CAG GTG CCA CAC CAG TCC AAC AGC ACC AAT ATC ITC
190 200 210 220
60
Phe Ser Pro Val Ser Ile Ala Thr Ala Phe Ala Met Leu Ser Leu
TTC TCC GGA GTG AGC ATC CCT ACA GCC TTT CCA ATG CTC TCC CTG
230 240 250 260 270
70 80
Gly Thr Lys Ala Asp Thr His Asp Glu Ile Leu Glu Gly Leu Asn
CGG ACC AAG GCT CAC ACT CAC GAT GAA ATC CTG GAG GGC CTG AAT
280 290 300 310
90
Phe Asn Leu Thr Glu Ile Pro Glu Ala Gln Ile His Glu Gly Phe
ITC AAC CTC ACG GAG ATT CCG GAC CCT CAC ATC CAT GAA CCC TTC
320 330 340 350 360
100 110
Gln Glu Leu Leu Arg Thr Leu Asn Gln Pro Asp Ser Gln Leu Gln
CAG GAA CTC CTC CCT ACC CTC AAC CAG CCA GAC AGC CAG CTC CAG
370 380 390 400
120
Leu Thr Thr Gly Asn Gly Leu Phe Leu Ser Glu Gly Leu Lys Leu
CTG ACC ACC CCC AAT GGC GTG TTC CTC AGC CAG GCC CTG AAC CTA
410 420 430 440 450
130 140
Val Asp Lys Phe Leu Glu Asp Val Lys Lys Leu Tyr His Ser Glu
GTG CAT AAG TTT ITG GAG GAT CTT AAA AAG TTG TAC CAC TCA GAA
460 470 480 490

FIG. 1B - page 2

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                                                                150
Ala Phe Thr Val Asn Phe Gly Asp Thr Glu Glu Ala Lys Lys Gln
GCC TTC ACT GTC AAC TTC GGG GAC ACC GAA GAG CCC AAG AAA CAG
   500          510          520          530          540

            160                                                 170
Ile Asn Asp Tyr Val Glu Lys Gly Thr Gln Gly Lys Ile Val Asp
ATC AAC GAT TAC GTG CAG AAG GGT ACT CAA CGC AAA ATT CTG GAT
          550          560          570          580

                                                                180
Leu Val Lys Glu Leu Asp Arg Asp Thr Val Phe Ala Leu Val Asn
TTG GTC AAC CAC CTT GAC AGA GAC ACA GTT TTT CCT CTC CTG AAT
   590          600          610          620          630

            190                                                 200
Tyr Ile Phe Phe Lys Gly Lys Trp Glu Arg Pro Phe Glu Val Lys
TAC ATC TTC TTT AAA GGC AAA TGG GAC ACA CCC TTT GAA CTC AAG
          640          650          660          670

                                                    210
Asp Thr Glu Glu Glu Asp Phe His Val Asp Gln Val Thr Thr Val
GAC ACC GAG GAA GAG GAC TTC CAC CTG GAC CAG GTG ACC ACC GTG
   680          690          700          710          720

            220                                                 230
Lys Val Pro Met Met Lys Arg Leu Gly Met Phe Asn Ile Gln His
AAG CTC CCT ATG ATG AAG CCT TTA CCC ATC TTT AAC ATC CAG CAT
          730          740          750          760

                                                    240
Cys Lys Lys Leu Ser Ser Trp Val Leu Leu Met Lys Tyr Leu Gly
TGT AAG AAG CTG TCC ACC TCC GTG CTG CTG ATC AAA TAC CTG GGC
   770          780          790          800          810

            250                                                 260
Asn Ala Thr Ala Ile Phe Phe Leu Pro Asp Glu Gly Lys Leu Gln
AAT GCC ACC GCC ATC TTC TTC CTG CCT GAT GAG GGG AAA CTA CAC
          820          830          840          850

                                                    270
His Leu Glu Asn Glu Leu Thr His Asp Ile Ile Thr Lys Phe Leu
CAC CTG GAA AAT GAA CTC ACC CAC GAT ATC ATC ACC AAG TTC CTC
   860          870          880          890          900

            280                                                 290
Glu Asn Glu Asp Arg Arg Ser Ala Ser Leu His Leu Pro Lys Leu
GAA AAT GAA CAC AGA AGC TCT CCC AGC TTA CAT TTA CCC AAA CTG
          910          920          930          940

                                                    300
Ser Ile Thr Gly Thr Tyr Asp Leu Lys Ser Val Leu Gly Gln Leu
TCC ATT ACT GGA ACC TAT GAT GTG AAG AGC CTC CTA CGT CAA CTG
   950          960          970          980          990

            310                                                 320
Gly Ile Thr Lys Val Phe Ser Asn Gly Ala Asp Leu Ser Gly Val
GGG ATC ACT AAG GTC TTC ACC AAT GCC GCT GAC CTC TCC CGG GTC
         1000         1010         1020         1030
```

FIG. 1B - page 3

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                                                                330
Thr Glu Glu Ala Pro Leu Lys Leu Ser Lys Ala Val His Lys Ala
ACA GAG GAC CCA CCC CTG AAG GTC TCC AAC CCC CTG CAT AAG CCT
  1040          1050          1060          1070          1080
                340
Val Leu Thr Ile Asp Glu Lys Gly Thr Glu Ala Ala Gly Ala Met
GTG CTG ACC ATC GAC GAG AAA GGG ACT GAA GCT GCT GGG CCC ATG
     1090          1100          1110          1120

                                                360
Phe Leu Glu Ala Ile Pro Met Ser Ile Arg Pro Glu Val Lys Phe
TTT TTA GAG GCC ATA CCC ATC TCT ATC CGC CCC CAG GTC AAG TTC
  1130          1140          1150          1160          1170
                370                                         380
Asn Lys Pro Phe Val Phe Leu Met Ile Glu Gln Asn Thr Lys Ser
AAC AAA CCC TTT GTC TTC TTA ATG ATT GAA CAA AAT ACC AAG TCT
         1180          1190          1200          1220

                                                390         394
Pro Leu Phe Met Gly Lys Val Val Asn Pro Thr Gln Lys STOP
CCC CTC TTC ATG GGA AAA GTG GTG AAT CCC ACC CAA AAA TAA
  1220          1230          1240          1250

CTGCCTCTCGCTCCTCAACCCCCCCCCC3'
```

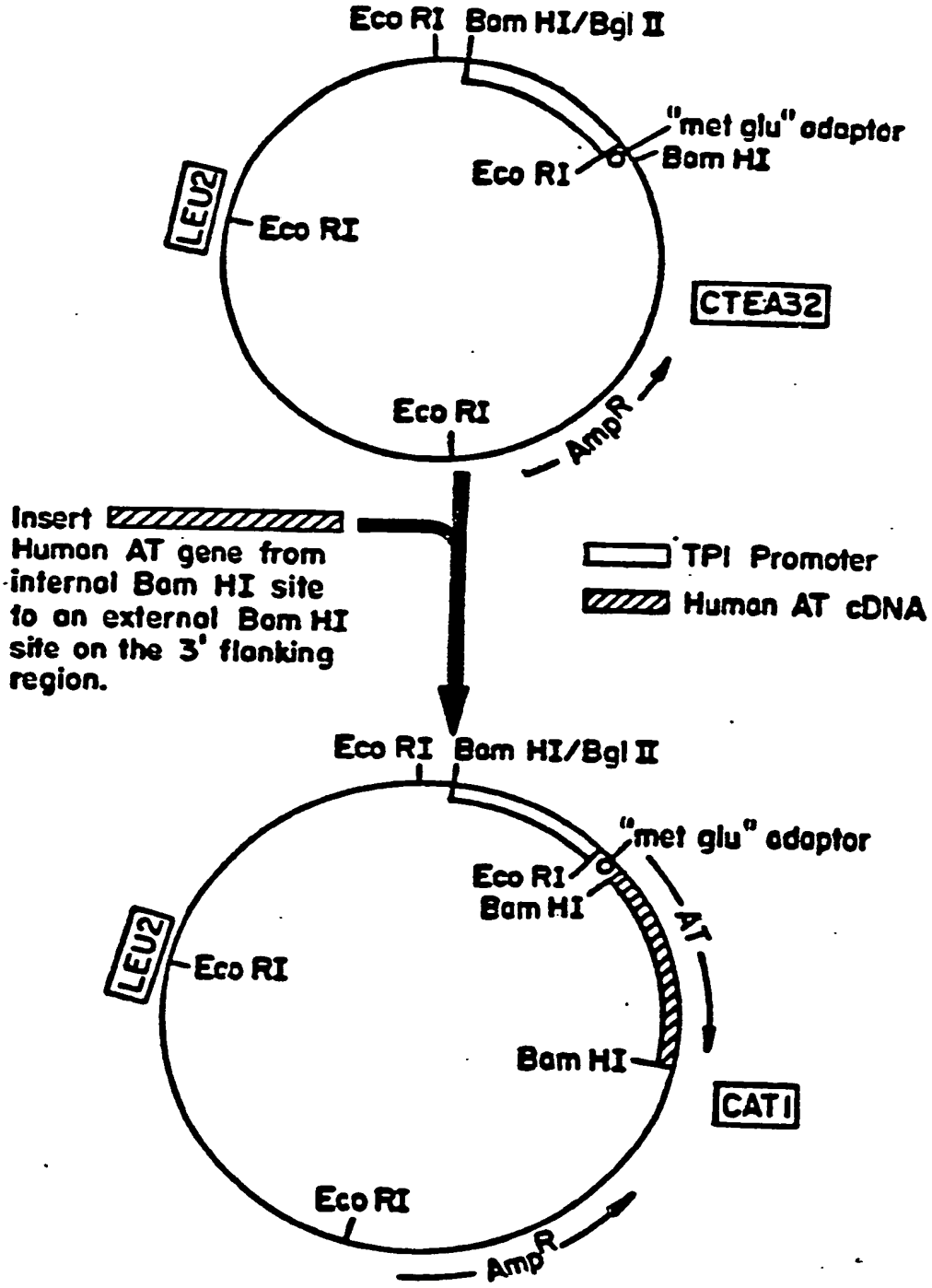
Eco RI
Bam HI/Bgl II
"met glu" adaptor
Bam HI
Eco RI
LEU2
Eco RI
CTEA32
Eco RI
AmpR
Insert
Human AT gene from internal Bam HI site to an external Bam HI site on the 3' flanking region.
TPI Promoter
Human AT cDNA
Eco RI
Bam HI/Bgl II
"met glu" adaptor
Eco RI
Bam HI
AT
LEU2
Eco RI
Bam HI
CAT I
Eco RI
AmpR

FIG. 2.

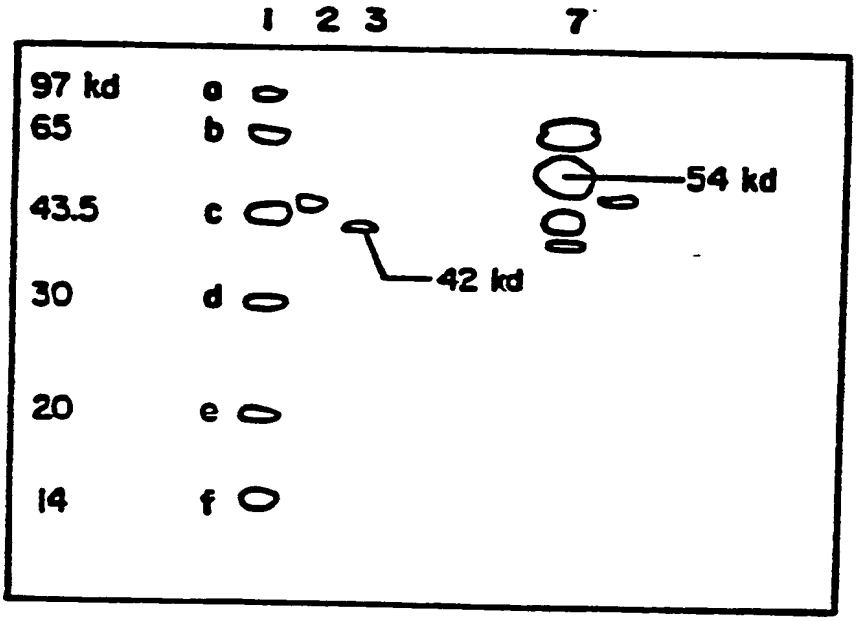

FIG. 3.

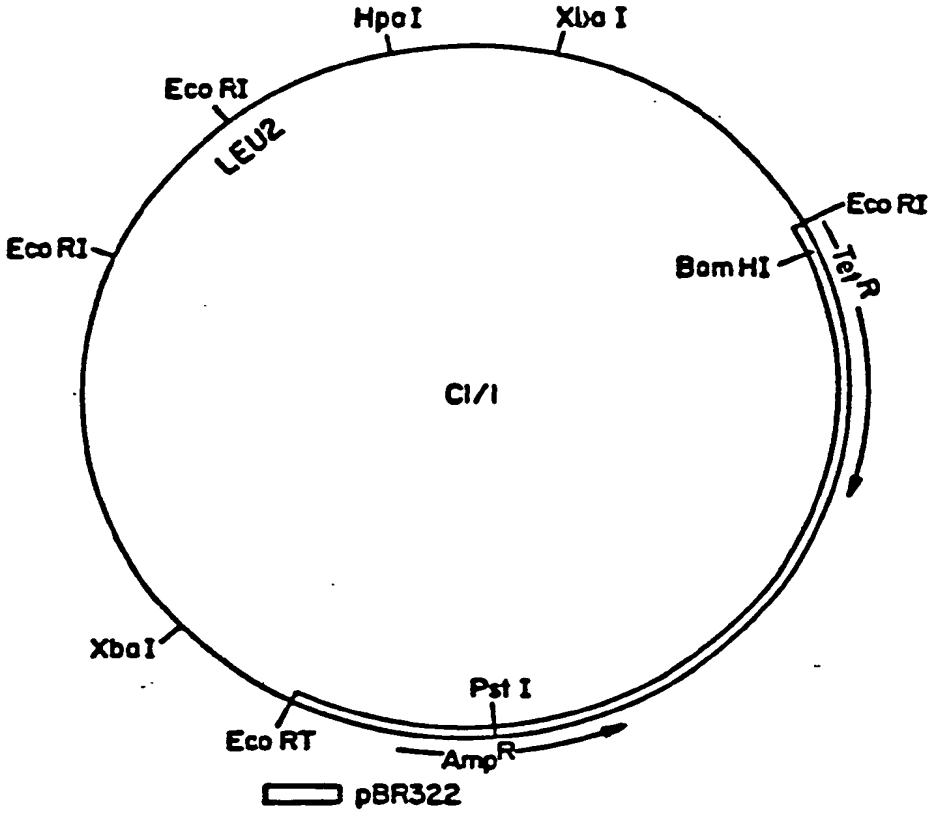
Hpa I
Xba I
Eco RI
LEU2
Eco RI
Eco RI
Bam HI
Tet R
C1/1
Xba I
Pst I
Eco RT
Amp R
pBR322

FIG. 4.

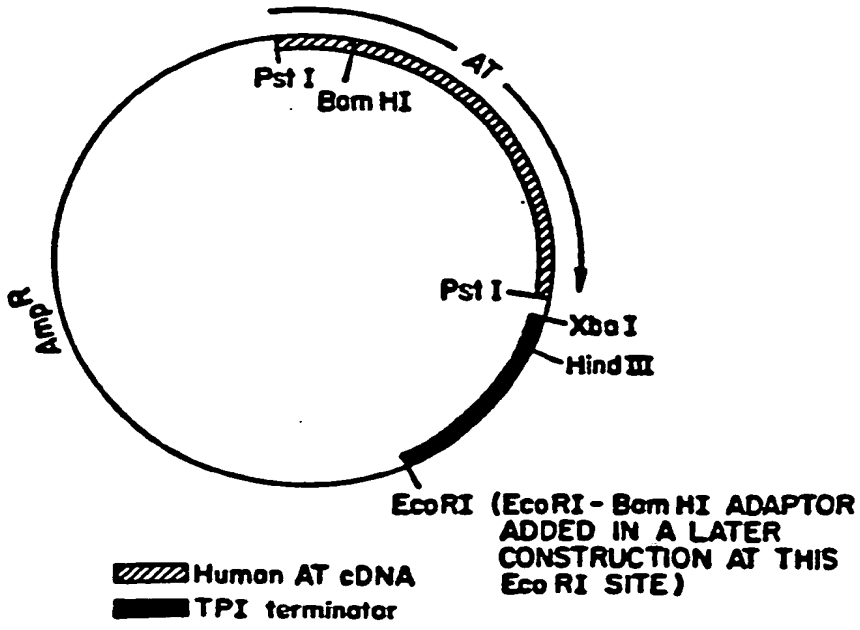
AT
Pst I
Bam HI
Pst I
Xba I
Hind III
Amp R
EcoRI (EcoRI - Bam HI ADAPTOR ADDED IN A LATER CONSTRUCTION AT THIS Eco RI SITE)
Human AT cDNA
TPI terminator

FIG. 6.

M13mp11/pUC13
1 2 3 4
THR MET ILE THR
ATG ACC ATG ATT ACG CCA AGC TTG GGC TGC AGG TCG ACT CTA GAG GAT CCC CGG GCG AGC TCG AAT TCA CTG GCC
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
PRO SER LEU GLY CYS ARG SER THR LEU GLU ASP PRO ARG ALA SER SER ASN SER LEU ALA
HindIII
PstI
SalI
AccI,HincII
XbaI
BamHI
XmaI
SmaI
SstI
EcoRI
HaeIII

FIG. 5.

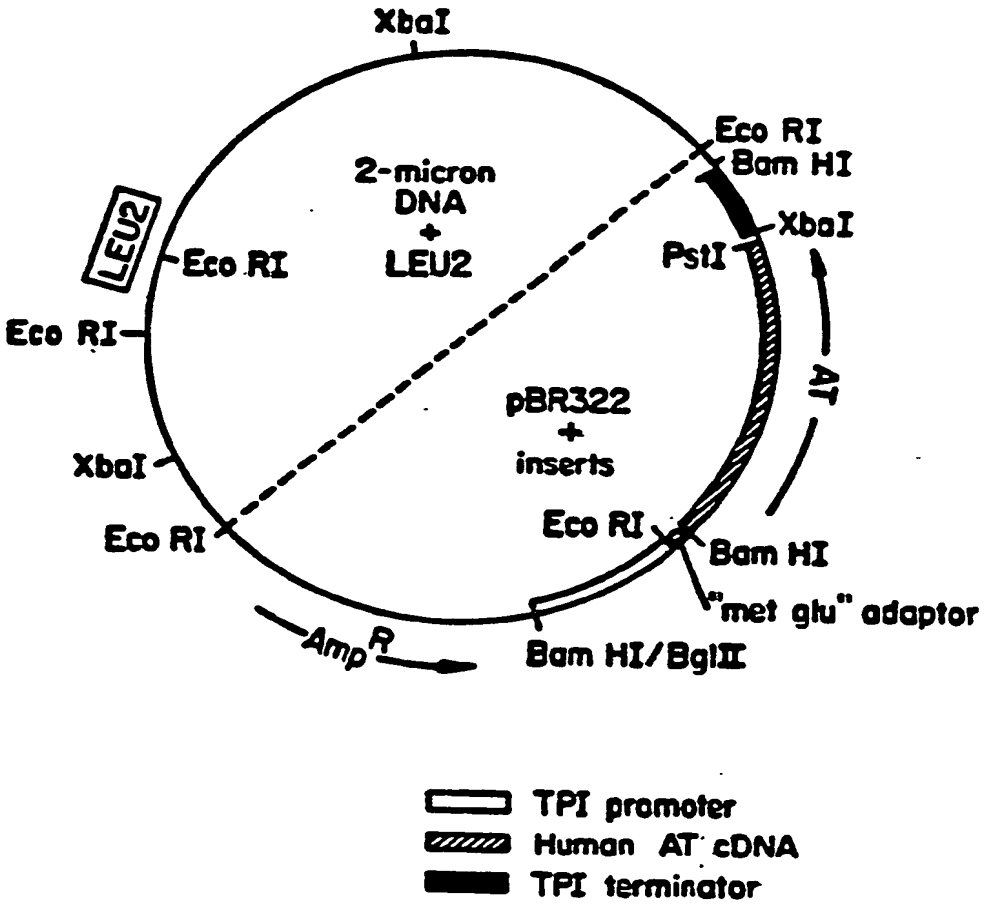
XbaI
Eco RI
Bam HI
XbaI
PstI
AT
2-micron
DNA
+
LEU2
LEU2
Eco RI
Eco RI
pBR322
+
inserts
XbaI
Eco RI
Eco RI
Bam HI
"met glu" adaptor
AmpR
Bam HI/BglII
TPI promoter
Human AT cDNA
TPI terminator

FIG. 7.